*SUPERCOILED CIRCULAR DNA-PROTEIN COMPLEX IN ESCHERICHIA COLI: PURIFICATION AND INDUCED CONVERSION TO AN OPEN CIRCULAR DNA FORM**

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Communicated by Charles Yanofsky, February 14, 1969

Abstract.—The 23*S* twisted circular form of *Col*E_1 DNA has been isolated from *Escherichia coli* as a tightly associated DNA-protein complex with a sedimentation coefficient of approximately 24*S*. Treatment of this complex with pronase, trypsin, sodium dodecyl sulfate, Sarkosyl, or heat results in a conversion to a slower sedimenting form of 17*S* or 18*S*, as determined by centrifugation in neutral sucrose gradients. These treatments do not alter the sedimentation properties of noncomplexed supercoiled *Col*E_1 DNA even in the presence of the *Col*E_1-protein complex. Electron microscopic analyses indicate that the decrease in sedimentation rate of the *Col*E_1-protein complex after treatment with these various agents is largely owing to an induced transition of *Col*E_1 DNA from the supercoiled to the open circular state.

Introduction.—Colicinogenic factor E_1 (*Col*E_1),§ a bacterial plasmid that determines the production of the antibiotic protein colicin E_1, has recently been isolated from *Escherichia coli* as a small, twisted circular double-stranded DNA molecule.[1] The circular molecule has a molecular weight of 4.2×10^6 and exhibits sedimentation coefficients at neutral pH of 23*S* and 17*S* for the twisted and open circular DNA forms, respectively.[1] As an approach to studying the mechanism and control of replication of extrachromosomal circular DNA elements, we have attempted to isolate naturally occurring *Col*E_1 DNA-protein complexes from gently lysed cells. The present communication describes the isolation of a supercoiled circular *Col*E_1 DNA-protein complex which can be induced to untwist and form an open circular double-stranded DNA molecule.

Materials and Methods.—Reagents: Sources were as follows: Brij 58, a nonionic detergent, Atlas Chemical; sodium deoxycholate, Mann Research Laboratories; sodium dodecyl sulfate, Fischer Scientific Co.; Sarkosyl NL30 (sodium dodecyl sarcosinate), Geigy Chemical Co.; pronase (B grade) and trypsin (pancreatic, crystalline, A grade), Calbiochem; egg-white lysozyme, Worthington Biochemical Corp.; bovine serum albumin (fraction V), Nutritional Biochemical Corp.; CsCl (technical grade), Penn Rare Metals Division of Kawecki Chemical Co.; ethidium bromide, a gift of the Boots Pure Drug Co., Ltd., Nottingham, England; [H^3]methyl-thymidine (6.7 c/mM), [H^3]methyl-thymine (18.2 c/mM), thymidine-2-[C^{14}](30 mc/mM), and carrier-free $H_3P^{32}O_4$, New England Nuclear Corp.

Strains: E. coli JC411 (*Col*E_1) has been described previously.[2] The *Col*E_1 factor was transferred from *E. coli* K-30. Noncolicinogenic JC411 was used as the *Col*E_1-negative control. The JC411 (*Col*E_1) strain was used also to prepare purified, noncomplexed P^{32}-labeled *Col*E_1 DNA. In cases where purified noncomplexed C^{14}-labeled *Col*E_1 DNA was used, a thymine-requiring strain of *E. coli* C600 (*Col*E_1) was utilized (requires thiamine, threonine, and leucine in addition to thymine for growth; isolated by M. Bazaral).

Medium: Tris-buffered medium contained per liter: 2.0 gm NH_4Cl, 5.0 gm NaCl, 0.37 gm KCl, 0.01 gm $MgCl_2 \cdot 6H_2O$, 0.02 gm Na_2SO_4, 5.0 gm casamino acids (Difco), 250 mg deoxyadenosine (Calbiochem, A grade), 250 mg thiamine (Calbiochem), 10 ml of 20% glycerol, and 100 ml of 1 M Tris, pH 7.3.

Normally, cells were grown in 30 ml of medium containing 0.1–0.3 mc of [H^3]thymine or [H^3]thymidine. When preparing purified noncomplexed *Col*E_1 DNA, the same medium was employed, except that 0.05 mc thymidine-2[C^{14}] or 1–5 mc of $H_3P^{32}O_4$ were used (in 30 ml) for radioisotope labeling, and deoxyadenosine was omitted.

Preparation of lysates: Lysates were prepared by a modification of the method of Sinsheimer and co-workers.[3, 4] Cells were harvested in log phase from 30 ml of culture and resuspended in 1 ml of cold 25% sucrose and 0.05 M Tris, pH 8. Lysozyme (0.2 ml of a 5 mg/ml solution in 0.25 M Tris, pH 8.0) was added, and after the suspension was maintained for 5 min at 0°C, 0.4 ml of EDTA (0.25 M, pH 8.0) was added. The suspension was kept at 0°C (swirling occasionally) for another 5 min after which lysis was brought about by adding 1.6 ml of a detergent mixture consisting of the following: 1% Brij 58, 0.4% sodium deoxycholate, 0.0625 M EDTA, and 0.05 M Tris, pH 8.0. After 5–10 min, the samples became relatively clear and viscous. They were then centrifuged at 2°C for 25 min at 48,000 g. This step normally pelleted about 95% of the H^3-labeled DNA. The supernatant, which contained the complexed *Col*E_1 DNA, is referred to as cleared lysate.

Sucrose density gradients: The 15–50% sucrose gradients contained 0.01 M EDTA, 0.06 M KCl, and 0.02 M Tris, pH 7.3. After centrifugation, the bottom of the centrifuge tube was punctured and 0.8-ml fractions were collected. A sample of 0.1 ml of each fraction was used for isotope counting. The 5–20% sucrose gradients were prepared in TES (0.05 M NaCl, 0.005 M EDTA, and 0.03 M Tris, pH 8.0). Fractions (10-drop) were collected from the bottom of the tube directly on filter papers for counting. All centrifugations were carried out in a Spinco model L2 or L4 ultracentrifuge under the conditions indicated.

Preparation of noncomplexed ColE$_1$ *DNA:* Purified noncomplexed *Col*E_1 was isolated by ethidium bromide–cesium chloride centrifugation of cleared lysates prepared as described above. Centrifugation conditions and the collection of the sample were carried out as described below. The recovered satellite *Col*E_1 DNA was dialyzed against 0.1 × SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.3) prior to use. This 23S twisted circular DNA exhibited properties identical to that of 23S DNA obtained from DNA preparations that were deproteinized prior to centrifugation.

Treatment of ColE$_1$ *24S complex:* The *Col*E_1 24S complex, purified by centrifugation in a 15–50% sucrose gradient, was dialyzed against, or diluted five- to eightfold, with TES, SSC, or 0.1 × SSC and mixed with the purified, noncomplexed *Col*E_1 DNA. The substance used for the treatment was added at the indicated concentration and the mixture was incubated at 25°C for 10 min. The final volume of the reaction mixture was 0.4 ml and normally contained 0.01–0.10 μg of *Col*E_1 DNA in the complexed form and a similar amount of noncomplexed *Col*E_1 DNA. After the 10-min incubation period, the sample immediately was layered on a preformed 5–20% sucrose gradient for centrifugation. In the case of the heat treatment, the mixture of complexed and noncomplexed *Col*E_1 DNA was incubated at 60°C for 20 min, quickly chilled, and then applied to the sucrose gradient.

Dye–buoyant density equilibrium centrifugation: Centrifugation was performed, using a modification of the method of Bauer and Vinograd,[5] on a Spinco model L4 ultracentrifuge in a type Ti-60 fixed-angle rotor at 44,000 rpm and 15°C for 48–60 hr. Each polyallomer centrifuge tube contained 15 gm CsCl, 12 ml of sample in TES, and 3 ml of a 1 mg/ml solution of ethidium bromide in TES. The remainder of the tube was filled with light mineral oil. At the end of the run, the bottom of the tube was punctured and 0.25–0.30-ml fractions were collected.

Counting of radioisotope: Samples were spotted or collected on 1-in. square filter papers, immersed in cold 10% trichloroacetic acid, ethanol, and then ether, and counted in a Beckman liquid scintillation counter, as previously described.[6] In experiments involving 5–20% sucrose density gradients, the ten-drop fractions were allowed to dry under an infrared bulb and counted directly.

Electron microscopy: Samples were prepared for electron microscopy by a modification of the Kleinschmidt and Zahn technique[7] as previously described.[8] Treated and untreated *Col*E_1 DNA-protein complex were spread directly after recovery from sucrose density gradients.

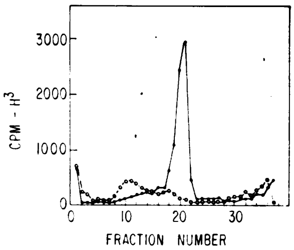

FIG. 1.—Sucrose gradient analyses of cleared lysates of strains JC411 (*ColE*$_1$) and JC411 (a noncolicinogenic strain). Gradients were 15–50% neutral sucrose and the material was sedimented (from right to left) in an SW25.1 rotor at 25,000 rpm for 15 hr, at 2°C. (●——●) JC411 (*ColE*$_1$); (O——O) JC411.

Results.—Identification of ColE$_1$ *DNA in cell lysates:* When cleared lysates from JC411 and JC411 (*Col*E_1) cells were centrifuged on 15–50 per cent neutral sucrose density gradients, the DNA profiles illustrated in Figure 1 were obtained. A very prominent peak of DNA labeled with H^3-thymine was observed near the middle of the gradient in the case of the JC411 (*Col*E_1) lysate. This DNA is absent in the lysate of the noncolicinogenic JC411 strain. When a sample of the material in this peak was examined by electron microscopy (Fig. 2*A*), approximately 90 per cent of the DNA molecules were in the form of double-stranded supercoiled (twisted) circles with a size and appearance characteristic of purified covalently closed circular *Col*E_1 DNA.[8]

Partial characterization of the ColE$_1$ *obtained from cell lysates:* Fractions from the *Col*E_1 DNA peak were pooled and either dialyzed or diluted, as described in *Materials and Methods*, to lower the sucrose concentration. This material was then mixed with P^{32}-labeled *Col*E_1 twisted circular DNA (prepared by the preparative dye–buoyant density technique), and the mixture was centrifuged on a 5–20 per cent sucrose density gradient. As shown in Figure 3*A*, the *Col*E_1 DNA obtained from the lysate sediments slightly faster than the added P^{32}-labeled 23*S* *Col*E_1 DNA and exhibits a sedimentation coefficient of approximately 24*S*.

The pooled 24*S* *Col*E_1 DNA tended to be unstable. Storage in TES at 5°C for several days normally resulted in a spontaneous shift to a slower-sedimenting form which is approximately 18*S*. Such a shift is not normally observed in *Col*E_1 supercoiled circular DNA preparations isolated by procedures involving dye–buoyant density centrifugation, or procedures involving deproteinization steps, except after prolonged storage[9] or limited treatment with dilute pancreatic endonuclease.[1, 9] Under the latter conditions the 23*S* twisted circular form of *Col*E_1 DNA is converted to a 17*S* open circular DNA form. The sedimentation profile of 24*S* *Col*E_1 DNA that had been purified from JC411 (*Col*E_1) and had spontaneously shifted to the slower-sedimenting, or 18*S*, form is shown in Figure 3*B*.

Since the observations up to this point indicated that the *Col*E_1 DNA, purified from JC411 (*Col*E_1) lysates as described, is complexed with a cellular substance, this material will subsequently be referred to as the *Col*E_1 24*S* complex.

Induction of ColE$_1$ *24S complex to a slower-sedimenting form by various agents known to affect protein structure:* The H^3-labeled *Col*E_1 24*S* complex was mixed

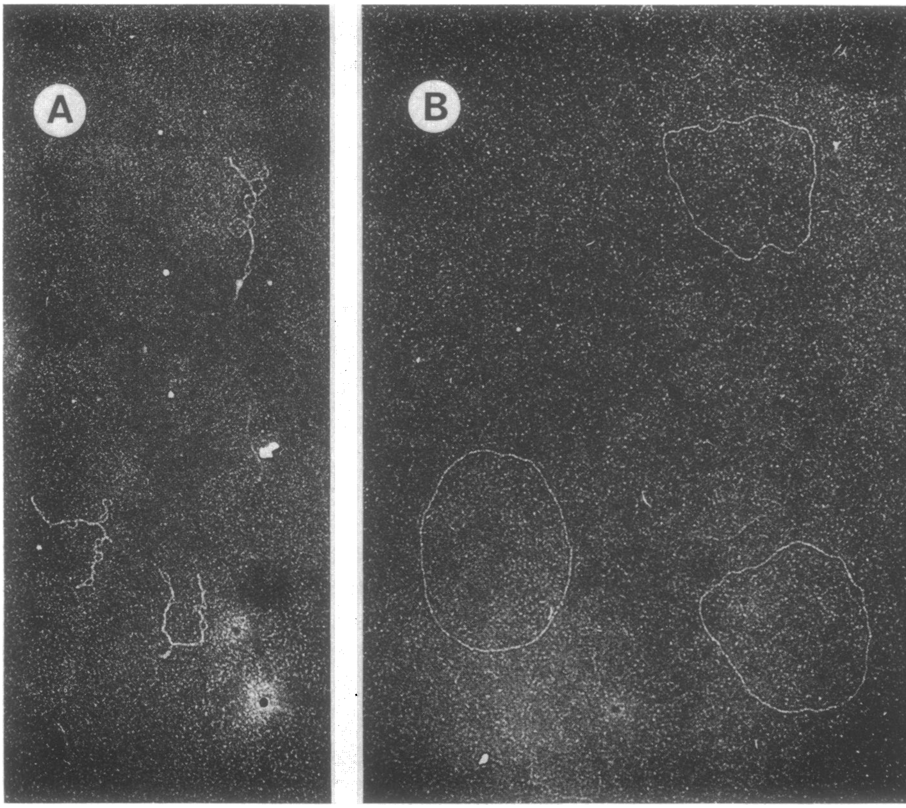

FIG. 2.—Electron micrographs taken of (*A*) *Col*E_1 DNA from a sucrose gradient peak similar to that shown in Fig. 1, and (*B*) *Col*E_1 DNA in the 17*S* peak obtained after treatment of a cleared lysate with pronase (1.6 mg/ml).

with noncomplexed C^{14}- or P^{32}-labeled 23*S* *Col*E_1 DNA and subjected to separate treatments with pronase, trypsin, bovine serum albumin, sodium dodecyl sulfate, Sarkosyl, and heat. The treated mixtures were then centrifuged on neutral sucrose density gradients. In the case of each of these treatments, except that

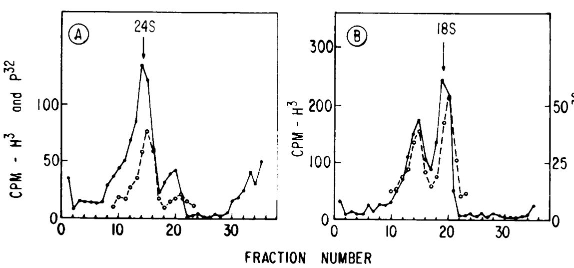

FIG. 3.—Sucrose gradient analysis of *Col*E_1 24*S* complex. Fractions of a peak similar to that shown in Fig. 1 were pooled and diluted, or dialyzed, as described in the text, mixed with noncomplexed P^{32}-labeled *Col*E_1 DNA (purified as described in *Materials and Methods*), layered on a 5–20% neutral sucrose density gradient, and centrifuged in the SW65 rotor at 50,000 rpm for 135 min at 15°C. Sedimentation was from right to left. (*A*) Freshly isolated *Col*E_1 24*S* complex. (*B*) *Col*E_1 24*S* complex that has partially undergone a spontaneous conversion to a slower-sedimenting form. The added noncomplexed P^{32}-labeled *Col*E_1 in (*B*) consisted of a mixture of 23*S* and 17*S* DNA.

(●——●) H^3-labeled *Col*E_1 complex; (○- - -○) P^{32}-labeled noncomplexed *Col*E_1 DNA.

involving incubation with bovine serum albumin, the *Col*E_1 24*S* complex was converted to a 17*S* or 18*S* form (Fig. 4). In no case did the added noncomplexed *Col*E_1 DNA undergo a similar conversion. In addition, in each case an identical mixture of *Col*E_1 24*S* complex and noncomplexed 23*S* *Col*E_1 DNA was simultaneously incubated under the same conditions, except that TES was substituted for the agent being tested. The control for the heat treatment consisted of incubating the mixture at 25°C. The simultaneously run controls did not undergo a conversion to the 17*S* or 18*S* form. In addition, a detergent mixture of Brij 58 and sodium deoxycholate at concentrations identical to that used for cell lysis failed to induce the conversion.

To test the possibility that the *Col*E_1 24*S* complex forms during the lysis procedure and is not present *in vivo*, noncomplexed 23*S* *Col*E_1 DNA was added directly to colicinogenic JC411 cells prior to, and after, lysis and then tested for susceptibility to conversion to the lower-sedimenting form by treatment with pronase. In every case the added *Col*E_1 DNA was resistant to conversion to the slower-sedimenting form.

In each case where the conditions induced a conversion to the slower-sedi-

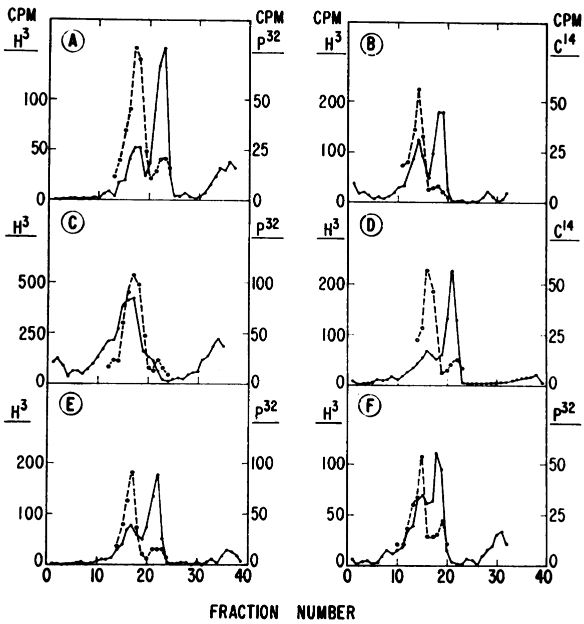

FIG. 4.—Sucrose gradient analyses of treated *Col*E_1 24*S* complex. Samples of the complex plus purified, noncomplexed *Col*E_1 DNA were centrifuged in a 5–20% neutral sucrose density gradient as described in Fig. 3, after prior treatment with (*A*) 1.25 mg/ml pronase (2.5 μg/ml produces the same result), (*B*) 250 μg/ml trypsin, (*C*) 250 μg/ml bovine serum albumin, (*D*) 0.25% sodium dodecyl sulfate, (*E*) 0.25% Sarkosyl, and (*F*) heat for 20 min at 60°C. Conditions of treatment are described in *Materials and Methods*. The noncomplexed *Col*E_1 DNA consists largely of supercoiled circular DNA (23*S*) and a small proportion of open circular DNA (17*S*).

(●——●) H^3-labeled *Col*E_1 DNA complex; (O- - -O) P^{32}- or C^{14}-labeled noncomplexed *Col*E_1 DNA.

menting form, a significant portion of the *Col*E_1 DNA in the pooled complex resisted conversion. The routine result of a number of experiments with sodium dodecyl sulfate and pronase was that approximately 20–30 per cent of the DNA was found to be resistant to conversion. This suggests that a proportion of the purified *Col*E_1 supercoiled DNA is not in the same complex state as the majority of the *Col*E_1 DNA, or is not associated with any cellular material. It was also observed that resistant material consistently sediments at 23*S* rather than at 24*S*.

Characterization of the slower-sedimenting form: The approximate 17*S* or 18*S* sedimentation coefficient for the converted form suggested that the induced conversion may have involved a change from the supercoiled (covalently closed) state to an open circular (or nicked) state.[10, 11] To test this possibility, a cleared lysate of JC411 (*Col*E_1) was treated with pronase (1.6 mg/ml) for 15 minutes at 25°C and then centrifuged on a 15–50 per cent sucrose density gradient for 15 hours at 2°C. This treatment resulted in the conversion of the *Col*E_1 24*S* complex to the slower-sedimenting form, with the gradient exhibiting a profile containing the typically prominent 17*S* peak. Material from this peak was examined by electron microscopy. Approximately 80 per cent of the DNA molecules exhibited the open circular structure. Some typical circular forms are shown in Figure 2*B*. There was no apparent conversion to a linear DNA form.

Centrifugation of the *Col*E_1 24*S* complex in a dye–buoyant density gradient for 60 hours at either 15 or 2°C also resulted in a conversion of the *Col*E_1 DNA to the nonsupercoiled state. It is not yet clear whether the conversion is induced directly by components of the gradient, or is simply the result of a spontaneous shift during the period of centrifugation. Equivalent portions of untreated and pronase-treated samples of the *Col*E_1 DNA 24*S* complex were each centrifuged to equilibrium in an ethidium bromide–CsCl gradient. As shown in Figure 5, approximately 30 per cent of the *Col*E_1 DNA was found, in both cases, as heavy satellite peaks in the position of covalently closed circular DNA.[1] This super-

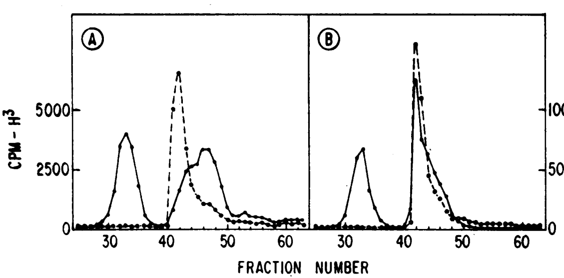

FIG. 5.—Dye–buoyant density gradient analyses of untreated and pronase-treated 24*S* *Col*E_1 DNA complex. *Col*E_1 24*S* complex purified by a 15–50% sucrose gradient centrifugation was treated with pronase (1.25 mg/ml) for 15 min at 25°C (final volume of 3.0 ml), while an identical amount of *Col*E_1 24*S* complex served as a control, in which case TES was substituted for pronase. The samples were then submitted to dye–buoyant density centrifugation as described in *Materials and Methods*. P^{32}-labeled *E. coli* chromosomal DNA (150 μg), extracted with phenol,[8] was included to serve as a marker for the position of DNA that is not in a covalently closed circular state. In addition, bovine serum albumin (2 mg/ml) was added to prevent nonspecific binding of the complex to the centrifuge tube wall. Centrifugation conditions and the collection of samples are described in *Materials and Methods*. Fractions were collected directly on filter paper for counting.

(*A*) Untreated *Col*E_1 24*S* complex; (*B*) pronase-treated complex; (●——●) *Col*E_1 DNA; (○- - -○) *E. coli* chromosomal DNA.

coiled DNA peak probably represents that fraction of *Col*E_1 DNA which is resistant to conversion. When this supercoiled DNA was recovered and treated with pronase, or sodium dodecyl sulfate in other experiments, it was found to resist conversion to the open circular state. In the case of the untreated *Col*E_1 24*S* complex, the open circular *Col*E_1 DNA that appears during the centrifugation procedure is found to band in a region less dense than that in which the chromosomal linear DNA marker is found to band. This difference is interpreted as resulting from the presence of tightly bound protein, since the banding position of the covalently open *Col*E_1 DNA was not distinguishable from the linear chromosomal DNA marker when the complex was treated with pronase. A similar DNA-protein association is not evident with the covalently closed circles surviving the centrifugation, since the banding position of this DNA relative to the chromosomal DNA marker is unchanged after pronase treatment.

Discussion.—*Col*E_1 DNA has been purified from gently prepared lysates as a 24*S* complex that consists of supercoiled *Col*E_1 DNA plus protein material. Association of *Col*E_1 DNA with protein is indicated by: (1) agents and conditions known to affect protein structure exert a marked effect on the structure of the complexed *Col*E_1 DNA but do not affect the structure of noncomplexed *Col*E_1 DNA; and (2) centrifugation of the *Col*E_1 24*S* complex in an ethidium bromide–CsCl gradient results in a transition to an open circular DNA form that is of a lighter buoyant density than deproteinized *E. coli* chromosomal DNA, and this density difference is abolished by pronase treatment of the 24*S* complex prior to centrifugation. Furthermore, preliminary data indicate that *Col*E_1 DNA prepared from a logarithmically growing JC411 (*Col*E_1) culture that has been incubated with chloramphenicol for 60 minutes is complexed to a significantly lesser extent than *Col*E_1 DNA isolated from untreated cultures. Under these conditions of chloramphenicol inhibition of protein synthesis, *Col*E_1 DNA synthesis continues to some extent.

A strong association between the protein material and *Col*E_1 DNA is indicated both by the inability of noncomplexed *Col*E_1 DNA to compete for the protein in the complex, and by the failure of centrifugation in an ethidium bromide–CsCl gradient to remove all the protein from the DNA. Attempts to remove protein from the *Col*E_1 24*S* complex by treatment with phenol normally resulted in low yields of DNA; however, when significant yields were obtained, analysis of the resulting DNA indicated that the majority of DNA was converted to the open circular form as a result of the phenol treatment.

The induced conversion to the slower-sedimenting (open circular) form of DNA is accompanied by removal of the majority, or all, of the protein in the case of treatment with pronase (see Fig. 5). On the other hand, material treated with sodium dodecyl sulfate or Sarkosyl probably contains some bound protein since it exhibits a lighter buoyant density than deproteinized chromosomal DNA (unpublished observation).

The nature of the protein-DNA association in the 24*S* complex is unknown. It is possible that one of the strands of the *Col*E_1 DNA is a covalently closed circle, while the other contains a nick, or gap, but is topologically restrained by one or more proteins which are tightly associated at the site of the gap. The involvement of a protein linker that covalently closes the break in the DNA strand seems unlikely since conversion to the open circular form can be induced by detergents and a short exposure to heat. An attractive alternative is that both DNA strands are covalently closed and the protein bound to the *Col*E_1 DNA includes an inactive endonuclease which is activated by the various treatments that induce an opening of the twisted structure. The activation could be the result of the preferential removal of a second protein, or subunit, that specifically represses the endonuclease, or the preferential inactivation of a region of the endonuclease that allosterically represses the catalytic activity. Detergents and proteases have been shown in several cases to alter the allosteric properties of an enzyme.[12–14] The physiological significance of the *Col*E_1 DNA-protein complex is presently under investigation. An endonuclease-catalyzed opening of a covalently closed circle may be one of the initial steps in the semiconservative duplication of closed circular DNA. Evidence has been obtained for the required attachment of parental DNA of bacteriophage ΦX-174 to a cellular structural component for semiconservative replication of this DNA.[15] Examination of the extrachromosomal circular DNA elements, *Col*E_2 and *Col*E_3, has indicated that similar DNA-protein complexes exist in these cases. Other bacterial plasmids and episomes are also being examined for similar specific associations with protein material.

We are indebted to Miss Joan Gaines and Mrs. Evelyn Johnson for their invaluable technical assistance.

* Supported by U.S. Public Health Service research grant AI-07194 and National Science Foundation grant GB-6297.

† Postdoctoral fellow of the U.S. Public Health Service, National Cancer Institute (5-F2-CA-36,754-02).

‡ U.S. Public Health Service Research Career Development Awardee KO4-6M07821.

§ Abbreviations used: *Col*E_1, colicinogenic factor E_1; Sarkosyl NL30, sodium dodecyl sarcosinate; TES, 0.05 *M* NaCl, 0.005 *M* EDTA, and 0.03 *M* Tris, pH 8.0; SSC, 0.15 *M* NaCl, 0.015 *M* sodium citrate, pH 7.3.

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